

**ANTISENSE MODULATION OF FLIP-C EXPRESSION****5 INTRODUCTION**

This application is a continuation of U.S. Serial No. 09/666,269 filed September 20, 2000, which is herein incorporated by reference in its entirety.

**10 FIELD OF THE INVENTION**

The present invention provides compositions and methods for modulating the expression of FLIP-c. In particular, this invention relates to compounds, particularly oligonucleotides, specifically hybridizable  
15 with nucleic acids encoding FLIP-c. Such compounds have been shown to modulate the expression of FLIP-c.

**BACKGROUND OF THE INVENTION**

Apoptosis, or programmed cell death, is a naturally  
20 occurring process that has been strongly conserved during evolution to prevent uncontrolled cell proliferation. This form of cell suicide plays a crucial role in the development and maintenance of multicellular organisms by eliminating superfluous or unwanted cells. However, if  
25 this process goes awry, excessive apoptosis results in cell loss and degenerative disorders including neurological disorders such as Alzheimers, Parkinsons, ALS, retinitis pigmentosa and blood cell disorders, while insufficient apoptosis contributes to the development of cancer,  
30 autoimmune disorders and viral infections (Konopleva et al., *Adv. Exp. Med. Biol.*, 1999, 457, 217-236).

Several stimuli can induce apoptosis, and recently, major advances have been made in understanding the signaling pathways mediated by the cell surface cytokine  
35 receptors activated by these stimuli, TNFR-1 and CD95 (Fas/APO-1).

The pathways leading from these receptors involve a proteolytic cascade orchestrated by a family of enzymes known as caspases (Thornberry, *Br. Med. Bull.*, **1997**, 53, 478-490). The most upstream caspase identified to date is  
5 caspase 8 (also known as CAP4, FLICE, MACH and Mch5). Caspase 8 is ubiquitously expressed in both fetal and adult tissues, with the exception of fetal brain and when overexpressed, induces apoptosis (Muzio et al., *Cell*, **1996**, 85, 817-827).

10 Caspase 8 interacts with the CD95 receptor in association with the adapter protein, FADD, through a previously identified protein motif contained within both proteins known as the death domain (Muzio et al., *Cell*,  
15 **1996**, 85, 817-827). Once recruited to the death-inducing signaling complex (DISC), caspase 8 undergoes autoproteolytic cleavage and subsequent activation (Martin et al., *J. Biol. Chem.*, **1998**, 273, 4345-4349; Medema et al., *Embo J.*, **1997**, 16, 2794-2804; Muzio et al., *J. Biol. Chem.*, **1998**, 273, 2926-2930; Srinivasula et al., *Proc.*  
20 *Natl. Acad. Sci. U. S. A.*, **1996**, 93, 14486-14491). While downstream effector caspases have been shown to cleave several classes of protein substrates, having somewhat redundant roles, upstream caspases such as caspase 8 function primarily to cleave and activate caspases  
25 downstream of receptor activation. One exception is cytosolic phospholipase A2. Caspase 8 has also been shown to cleave this non-caspase proinflammatory enzyme (Luschen et al., *Biochem. Biophys. Res. Commun.*, **1998**, 253, 92-98).

It has recently been demonstrated that caspase 8 can  
30 be activated by several death receptor-independent pathways as well. Caspase 8 can be activated by anticancer drugs in the absence of CD95 receptor activation in human leukemic T-cell lines suggesting the presence of an alternate apoptotic pathway (Bantel et al., *Cancer Res.*, **1999**, 59,  
35 2083-2090; Wesselborg et al., *Blood*, **1999**, 93, 3053-3063).

Medema et al. have also demonstrated that caspase 8 is cleaved by granzyme B in HeLa cells, indicating its involvement in perforin-induced apoptosis, another CD95-independent apoptotic pathway (Medema et al., *Eur. J.*

5 *Immunol.*, **1997**, 27, 3492-3498). Other CD95-independent pathways include mediation by nitric-oxide (Chlichlia et al., *Blood*, **1998**, 91, 4311-4320), cytochrome c (Kuwana et al., *J. Biol. Chem.*, **1998**, 273, 16589-16594), and the Sendai virus (Bitzer et al., *J. Virol.*, **1999**, 73, 702-708).

10 Caspase 8 represents a potential therapeutic target in several diseases including AIDS and AIDS-related disorders. It has been shown to be upregulated by the AIDS viral Tat protein (Bartz and Emerman, *J. Virol.*, **1999**, 73, 1956-1963; Peter et al., *Br. Med. Bull.*, **1997**, 53, 604-616).

15 Mandruzzato et al. identified an antigen recognized by cytolytic T lymphocytes encoded by a mutated form of the caspase 8 gene in head and neck carcinoma cells. This mutation, found only in the tumor cells, alters the stop codon thereby adding 88 amino acids to the protein and  
20 reducing the activity of the caspase (Mandruzzato et al., *J. Exp. Med.*, **1997**, 186, 785-793).

It is currently believed that modulation of caspase 8 function represents a potential therapeutic target in a variety of deregulated apoptotic pathologic conditions.

25 FLIP-c is a natural dominant negative regulator of caspase 8. It acts as an anti-apoptotic protein and therefore represents a very specific avenue of therapeutic intervention in the regulation of caspase activation.

Isolation and characterization of FLIP-c has been  
30 carried out by many investigators and therefore the protein has several designations. FLIP-c is also known as FLIP (Irmeler et al., *Nature*, **1997**, 388, 190-195), Casper (Shu et al., *Immunity*, **1997**, 6, 751-763), I-Fllice (Hu et al., *J. Biol. Chem.*, **1997**, 272, 17255-17257), FLAME-1 (Srinivasula  
35 et al., *J. Biol. Chem.*, **1997**, 272, 18542-18545), CASH

(Goltsev et al., *J. Biol. Chem.*, **1997**, 272, 19641-19644), CLARP (Inohara et al., *Proc. Natl. Acad. Sci. U. S. A.*, **1997**, 94, 10717-10722), MRIT (Han et al., *Proc. Natl. Acad. Sci. U. S. A.*, **1997**, 94, 11333-11338) and Usurpin (Rasper et al., *Cell Death Differ.*, **1998**, 5, 271-288). Disclosed in the US Patent 6,037, 461 and in the corresponding PCT Publication WO 98/52963 are the polynucleotide encoding FLIP-c as well as the polypeptide, antibodies to the protein and a composition comprising the nucleic acid and a pharmaceutically acceptable carrier. Also disclosed are recombinant expression vectors encoding FLIP-c and host cells comprising said vector (Alnemri, **2000**; Alnemri, **1998**). Antisense oligonucleotides are generally disclosed. Disclosed in the PCT Publication WO 00/03023 are recombinant DNA molecules encoding FLIP-c and fragments thereof as well as vectors encoding said DNA and host cells containing said vectors and DNA that hybridizes to DNA encoding FLIP-c. Also disclosed are methods for the identification of inhibitors of the interaction between FLIP-c and caspase 8 (Nicholson et al., **2000**). The polynucleotide and polypeptide encoding FLIP-c is also disclosed in the PCT Publication WO 98/44103 in addition to antibodies to the protein and a method of making the protein for use as an inhibitor of apoptosis (Krammer et al., **1998**).

The protein is found in two forms in the cell, a short and a long form resulting from alternative splicing of the mRNA (Goltsev et al., *J. Biol. Chem.*, **1997**, 272, 19641-19644; Han et al., *Proc. Natl. Acad. Sci. U. S. A.*, **1997**, 94, 11333-11338; Irmeler et al., *Nature*, **1997**, 388, 190-195; Rasper et al., *Cell Death Differ.*, **1998**, 5, 271-288; Shu et al., *Immunity*, **1997**, 6, 751-763). The long form of FLIP-c is upregulated upon cross-linking of the B cell antigen receptor which induces resistance to apoptosis (Wang et

al., *Eur. J. Immunol.*, **2000**, 30, 155-163). Other studies have also implicated the level of the long form as a primary determinant of susceptibility to Fas-mediated apoptosis (Tepper and Seldin, *Blood*, **1999**, 94, 1727-1737).

5 Ectopic expression of the long form in transformed keratinocytes by transfection with a FLIP-c expression vector resulted in resistance to TRAIL-mediated apoptosis in these cells (Leverkus et al., *Cancer Res.*, **2000**, 60, 553-559).

10 In the cell, FLIP-c plays a role in death pathways initiated by tumor necrosis alpha (TNF) and Fas reviewed in (Scaffidi et al., *J. Biol. Chem.*, **1999**, 274, 1541-1548; Tschoopp et al., *Curr. Opin. Immunol.*, **1998**, 10, 552-558). In doing so, FLIP-c interacts with several other death  
15 pathway proteins including FADD, caspase 8, caspase 3, Bcl-2 family members, TRAF1 and TRAF2 (Goltsev et al., *J. Biol. Chem.*, **1997**, 272, 19641-19644; Han et al., *Proc. Natl. Acad. Sci. U. S. A.*, **1997**, 94, 11333-11338; Hu et al., *J. Biol. Chem.*, **1997**, 272, 17255-17257; Rasper et al., *Cell*  
20 *Death Differ.*, **1998**, 5, 271-288; Shu et al., *Immunity*, **1997**, 6, 751-763; Srinivasula et al., *J. Biol. Chem.*, **1997**, 272, 18542-18545).

Expression of FLIP-c is found in most tissues with the highest levels in the heart, placenta, spleen, leukocytes,  
25 testes and skeletal muscle (Hu et al., *J. Biol. Chem.*, **1997**, 272, 17255-17257; Irmeler et al., *Nature*, **1997**, 388, 190-195). Expression has also been demonstrated in metastatic cutaneous melanoma lesions in patients, with no expression in surrounding normal skin. FLIP-c has been  
30 shown to protect lymphoma cell lines against death receptor-induced apoptosis (Irmeler et al., *Nature*, **1997**, 388, 190-195) and to protect monocytes and macrophages from Fas-mediated apoptosis suggesting that FLIP-c may play a

major role in inflammation (Perlman et al., *J. Exp. Med.*,  
1999, 190, 1679-1688).

Furthermore, FLIP-c expression levels have been shown  
to be downregulated in medial smooth muscle cells after  
5 balloon angioplasty and is absent in the atherosclerotic  
plaque. It is consequently believed that FLIP-c  
participates in the pathways mediating control of viability  
of the cells of the atherosclerotic intima (Imanishi et al.,  
*Am. J. Pathol.*, 2000, 156, 125-137). Disclosed in the PCT  
10 Publication WO 99/42570 are methods for treating conditions  
associated with vascular wall inflammation, particularly  
atherosclerosis and vascular injury involving administering  
to subjects in need of such treatment an effective amount  
of a FLIP-c molecule (Walsh, 1999). Isolated nucleic acid  
15 molecules encoding FLIP-c polypeptides or fragments thereof  
are disclosed, as are complements thereof.

Que et al. demonstrated in cholangiocarcinoma cells  
that FLIP-c antisense treatment by stable transfection with  
complementary DNA for FLIP-c in the reverse orientation  
20 reduced FLIP-c protein expression by 90% and increased Fas-  
mediated apoptosis 2-fold suggesting that inhibition of  
FLIP-c may aid in the treatment of cholangiocarcinoma (Que  
et al., *Hepatology*, 1999, 30, 1398-1404).

Currently, there are no known therapeutic agents which  
25 effectively inhibit the synthesis of FLIP-c and to date,  
strategies aimed at modulating FLIP-c function have  
involved the use of antibodies, caspase inhibitors,  
antisense expression vectors and molecules that block  
upstream entities such as the death receptors.  
30 Consequently, there remains a long felt need for agents  
capable of effectively inhibiting FLIP-c function.

Antisense technology is emerging as an effective means  
for reducing the expression of specific gene products and  
may therefore prove to be uniquely useful in a number of  
35 therapeutic, diagnostic, and research applications for the  
modulation of FLIP-c expression.

The present invention provides compositions and methods for modulating FLIP-c expression, including modulation of both the long and short isoforms of FLIP-c.

## 5 SUMMARY OF THE INVENTION

The present invention is directed to compounds, particularly antisense oligonucleotides, which are targeted to a nucleic acid encoding FLIP-c, and which modulate the expression of FLIP-c. Pharmaceutical and other  
10 compositions comprising the compounds of the invention are also provided. Further provided are methods of modulating the expression of FLIP-c in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds or compositions of the invention.  
15 Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of FLIP-c by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds  
20 or compositions of the invention.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric compounds, particularly antisense oligonucleotides, for use in  
25 modulating the function of nucleic acid molecules encoding FLIP-c, ultimately modulating the amount of FLIP-c produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding FLIP-c. As used herein, the terms  
30 "target nucleic acid" and "nucleic acid encoding FLIP-c" encompass DNA encoding FLIP-c, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the  
35 normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as

"antisense". The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of FLIP-c. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding FLIP-c. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the



"AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function in vivo. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used in vivo to initiate translation of an mRNA molecule transcribed from a gene encoding FLIP-c, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted

effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including

5 nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus  
10 including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an  
15 mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as  
20 "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target  
25 regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It  
30 has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently  
35 complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds.

"Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment, and in the case of in vitro assays, under conditions in which the assays are performed.

Antisense and other compounds of the invention which hybridize to the target and inhibit expression of the

target are identified through experimentation, and the sequences of these compounds are hereinbelow identified as preferred embodiments of the invention. The target sites to which these preferred sequences are complementary are  
5 hereinbelow referred to as "active sites" and are therefore preferred sites for targeting. Therefore another embodiment of the invention encompasses compounds which hybridize to these active sites.

Antisense compounds are commonly used as research  
10 reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to  
15 distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic  
20 uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to humans and numerous clinical trials are  
25 presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

In the context of this invention, the term  
30 "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as  
35 oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms

because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

5        While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this  
10       invention preferably comprise from about 8 to about 50 nucleobases (i.e. from about 8 to about 50 linked nucleosides). Particularly preferred antisense compounds are antisense oligonucleotides, even more preferably those comprising from about 12 to about 30 nucleobases. Antisense  
15       compounds include ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic RNAs or catalytic oligonucleotides which hybridize to the target nucleic acid and modulate its expression.

As is known in the art, a nucleoside is a base-sugar  
20       combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar  
25       portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form  
30       a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as  
35       forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this  
5 specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as  
10 sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkyl-  
15 phosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thiono-  
20 alkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage.  
25 Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts,  
30 mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808;  
4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897;  
35 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131;  
5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677;  
5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111;

5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

5 Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short  
10 chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones;  
15 methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH<sub>2</sub> component  
20 parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564;  
25 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, certain of which are commonly owned with this  
30 application, and each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups.  
35 The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has

been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., *Science*, **1991**, 254, 1497-1500.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular  $-\text{CH}_2-\text{NH}-\text{O}-\text{CH}_2-$ ,  $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{O}-\text{CH}_2-$  [known as a methylene (methylimino) or MMI backbone],  $-\text{CH}_2-\text{O}-\text{N}(\text{CH}_3)-\text{CH}_2-$ ,  $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{N}(\text{CH}_3)-\text{CH}_2-$  and  $-\text{O}-\text{N}(\text{CH}_3)-\text{CH}_2-\text{CH}_2-$  [wherein the native phosphodiester backbone is represented as  $-\text{O}-\text{P}-\text{O}-\text{CH}_2-$ ] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted  $\text{C}_1$  to  $\text{C}_{10}$  alkyl or  $\text{C}_2$  to  $\text{C}_{10}$  alkenyl and alkynyl. Particularly preferred are  $\text{O}[(\text{CH}_2)_n\text{O}]_m\text{CH}_3$ ,  $\text{O}(\text{CH}_2)_n\text{OCH}_3$ ,  $\text{O}(\text{CH}_2)_n\text{NH}_2$ ,  $\text{O}(\text{CH}_2)_n\text{CH}_3$ ,  $\text{O}(\text{CH}_2)_n\text{ONH}_2$ , and  $\text{O}(\text{CH}_2)_n\text{ON}[(\text{CH}_2)_n\text{CH}_3]_2$ , where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position:  $\text{C}_1$  to  $\text{C}_{10}$  lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl,



aralkyl, O-alkaryl or O-aralkyl, SH, SCH<sub>3</sub>, OCN, Cl, Br, CN, CF<sub>3</sub>, OCF<sub>3</sub>, SOCH<sub>3</sub>, SO<sub>2</sub>CH<sub>3</sub>, ONO<sub>2</sub>, NO<sub>2</sub>, N<sub>3</sub>, NH<sub>2</sub>, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, **1995**, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminoethoxy, i.e., a O(CH<sub>2</sub>)<sub>2</sub>ON(CH<sub>3</sub>)<sub>2</sub> group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'-O-CH<sub>2</sub>-O-CH<sub>2</sub>-N(CH<sub>2</sub>)<sub>2</sub>, also described in examples hereinbelow.

A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene (-CH<sub>2</sub>-)<sub>n</sub> group bridging the 2' oxygen atom and the 3' or 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

Other preferred modifications include 2'-methoxy (2'-O-CH<sub>3</sub>), 2'-aminopropoxy (2'-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>), 2'-allyl (2'-CH<sub>2</sub>-CH=CH<sub>2</sub>), 2'-O-alkyl (2'-O-CH<sub>2</sub>-CH=CH<sub>2</sub>) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents

that teach the preparation of such modified sugar structures include, but are not limited to, U.S.:

4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878;  
5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811;  
5 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053;  
5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and  
5,700,920, certain of which are commonly owned with the  
instant application, and each of which is herein  
incorporated by reference in its entirety.

10 Oligonucleotides may also include nucleobase (often  
referred to in the art simply as "base") modifications or  
substitutions. As used herein, "unmodified" or "natural"  
nucleobases include the purine bases adenine (A) and  
guanine (G), and the pyrimidine bases thymine (T), cytosine  
15 (C) and uracil (U). Modified nucleobases include other  
synthetic and natural nucleobases such as 5-methylcytosine  
(5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine,  
2-aminoadenine, 6-methyl and other alkyl derivatives of  
adenine and guanine, 2-propyl and other alkyl derivatives  
20 of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-  
thiocytosine, 5-halouracil and cytosine, 5-propynyl ( $-C\equiv C-$   
 $CH_3$ ) uracil and cytosine and other alkynyl derivatives of  
pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-  
uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-  
25 thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted  
adenines and guanines, 5-halo particularly 5-bromo, 5-  
trifluoromethyl and other 5-substituted uracils and cyto-  
sines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-  
amino-adenine, 8-azaguanine and 8-azaadenine, 7-  
30 deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-  
deazaadenine. Further modified nucleobases include  
tricyclic pyrimidines such as phenoxazine cytidine(1H-  
pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine  
cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one),  
35 G-clamps such as a substituted phenoxazine cytidine (e.g.  
9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-  
one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one),

pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyl-adenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121; 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, certain of which are commonly

owned with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

5           Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. The compounds of the  
10 invention can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the  
15 pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterol, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines,  
20 coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA. Groups that  
25 enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer uptake, distribution, metabolism or excretion. Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed  
30 October 23, 1992 the entire disclosure of which is incorporated herein by reference. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, **1989**,  
86, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Let.*, **1994**, 4, 1053-1060), a thioether, e.g., hexyl-  
35 S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*,

1992, 660, 306-309; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1993, 3, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, 1992, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-  
5 Behmoaras et al., *EMBO J.*, 1991, 10, 1111-1118; Kabanov et al., *FEBS Lett.*, 1990, 259, 327-330; Svinarchuk et al., *Biochimie*, 1993, 75, 49-54), a phospholipid, e.g., dihexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al.,  
10 *Tetrahedron Lett.*, 1995, 36, 3651-3654; Shea et al., *Nucl. Acids Res.*, 1990, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, 1995, 14, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651-3654),  
15 a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277, 923-937).

Oligonucleotides of the invention may also be conjugated to  
20 active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (S)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indo-  
25 methicin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic.

Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is incorporated herein by  
30 reference in its entirety.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717,

5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802;  
5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718;  
5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779;  
4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582;  
5 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830;  
5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506;  
5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241,  
5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667;  
5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481;  
10 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and  
5,688,941, certain of which are commonly owned with the  
instant application, and each of which is herein  
incorporated by reference.

It is not necessary for all positions in a given  
15 compound to be uniformly modified, and in fact more than  
one of the aforementioned modifications may be incorporated  
in a single compound or even at a single nucleoside within  
an oligonucleotide. The present invention also includes  
antisense compounds which are chimeric compounds.  
20 "Chimeric" antisense compounds or "chimeras," in the  
context of this invention, are antisense compounds,  
particularly oligonucleotides, which contain two or more  
chemically distinct regions, each made up of at least one  
monomer unit, i.e., a nucleotide in the case of an  
25 oligonucleotide compound. These oligonucleotides typically  
contain at least one region wherein the oligonucleotide is  
modified so as to confer upon the oligonucleotide increased  
resistance to nuclease degradation, increased cellular  
uptake, and/or increased binding affinity for the target  
30 nucleic acid. An additional region of the oligonucleotide  
may serve as a substrate for enzymes capable of cleaving  
RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is  
a cellular endonuclease which cleaves the RNA strand of an  
RNA:DNA duplex. Activation of RNase H, therefore, results  
35 in cleavage of the RNA target, thereby greatly enhancing  
the efficiency of oligonucleotide inhibition of gene  
expression. Consequently, comparable results can often be

obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely  
5 detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more  
10 oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid  
15 structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is  
20 herein incorporated by reference in its entirety.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors  
25 including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

30 The antisense compounds of the invention are synthesized in vitro and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the in vivo synthesis of antisense molecules.

35 The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as

for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such

5 uptake, distribution and/or absorption assisting

formulations include, but are not limited to, U.S.:

5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291;

5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330;

4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170;

10 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978;

5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259;

5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of

which is herein incorporated by reference.

The antisense compounds of the invention encompass any  
15 pharmaceutically acceptable salts, esters, or salts of such  
esters, or any other compound which, upon administration to  
an animal including a human, is capable of providing  
(directly or indirectly) the biologically active metabolite  
or residue thereof. Accordingly, for example, the  
20 disclosure is also drawn to prodrugs and pharmaceutically  
acceptable salts of the compounds of the invention,  
pharmaceutically acceptable salts of such prodrugs, and  
other bioequivalents.

The term "prodrug" indicates a therapeutic agent that  
25 is prepared in an inactive form that is converted to an  
active form (i.e., drug) within the body or cells thereof  
by the action of endogenous enzymes or other chemicals  
and/or conditions. In particular, prodrug versions of the  
oligonucleotides of the invention are prepared as SATE  
30 [(S-acetyl-2-thioethyl) phosphate] derivatives according to  
the methods disclosed in WO 93/24510 to Gosselin et al.,  
published December 9, 1993 or in WO 94/26764 and U.S.  
5,770,713 to Imbach et al.

The term "pharmaceutically acceptable salts" refers to  
35 physiologically and pharmaceutically acceptable salts of  
the compounds of the invention: i.e., salts that retain the



desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chlorprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, 1977, 66, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid,

lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, 5 nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 10 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of 15 cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and 20 include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not 25 limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric 30 acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic 35 acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed

from elemental anions such as chlorine, bromine, and iodine.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of FLIP-c is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding FLIP-c, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding FLIP-c can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of FLIP-c in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or

insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Preferred topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearoylphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). Oligonucleotides of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters include but are not limited arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprinate, tricaprinate, monoolein, dilaurin, glyceryl 1-monocaprinate, 1-dodecylazacycloheptan-2-one, an

acylcarnitine, an acylcholine, or a C<sub>1-10</sub> alkyl ester (e.g. isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in United States  
5 patent application 09/315,298 filed on May 20, 1999 which is incorporated herein by reference in its entirety.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-  
10 aqueous media, capsules, gel capsules, sachets, tablets or minitabets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Preferred oral formulations are those in which oligonucleotides of the invention are administered in  
15 conjunction with one or more penetration enhancers surfactants and chelators. Preferred surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Preferred bile acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic  
20 acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucolic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate, sodium glycodihydrofusidate, . Preferred fatty acids include arachidonic acid, undecanoic  
25 acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a  
30 diglyceride or a pharmaceutically acceptable salt thereof (e.g. sodium). Also preferred are combinations of penetration enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly preferred combination is the sodium salt of lauric acid, capric acid  
35 and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Oligonucleotides of the invention may be delivered

orally in granular form including sprayed dried particles, or complexed to form micro or nanoparticles.

Oligonucleotide complexing agents include

poly-amino acids; polyimines; polyacrylates;

5 polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG) and starches;

polyalkylcyanoacrylates; DEAE-derivatized polyimines, pollulans, celluloses and starches. Particularly preferred

10 complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylamino-methylethylene P(TDAE), polyaminostyrene (e.g. p-amino), poly(methylcyanoacrylate), poly(ethylcyanoacrylate),

15 poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcynaoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA),

20 alginate, and polyethyleneglycol (PEG). Oral formulations for oligonucleotides and their preparation are described in detail in United States applications 08/886,829 (filed July 1, 1997), 09/108,673 (filed July 1, 1998), 09/256,515 (filed February 23, 1999), 09/082,624 (filed May 21, 1998) and 09/315,298 (filed May 20, 1999) each of which is  
25 incorporated herein by reference in their entirety.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers,  
30 diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and  
35 liposome-containing formulations. These compositions may be generated from a variety of components that include, but

are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product. The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the present invention.

## Emulsions

The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1  $\mu$ m in diameter. (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi et al., in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex



formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a  
5 system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or  
10 discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style  
15 ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers,  
20 absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active  
25 agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*,  
30 Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the  
35 hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the

preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms*,

- 5 Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic  
10 properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous  
15 preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgate, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as  
20 carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants,  
25 hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel  
30 Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth),  
35 cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for

example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of reasons of ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are

among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: *Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988,

Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional  
5 emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions  
10 include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500),  
15 decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves  
20 to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-  
25 emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not  
30 limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils  
35 and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced

absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides et al., *Pharmaceutical Research*, **1994**, *11*, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, **1993**, *13*, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides et al., *Pharmaceutical Research*, **1994**, *11*, 1385; Ho et al., *J. Pharm. Sci.*, **1996**, *85*, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five

broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 92). Each of these classes has been discussed  
5 above.

### Liposomes

There are many organized surfactant structures besides microemulsions that have been studied and used for the  
10 formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term  
15 "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the  
20 composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages *in vivo*.

25 In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to  
30 pass through such fine pores.

Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect  
35 encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical*

*Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and  
5 the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the  
10 liposomes start to merge with the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

Liposomal formulations have been the focus of  
15 extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side-effects related to high systemic absorption of the  
20 administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

Several reports have detailed the ability of liposomes  
25 to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

30 Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized  
35 in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into



the cell cytoplasm (Wang et al., *Biochem. Biophys. Res. Commun.*, **1987**, 147, 980-985).

Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou et al., *Journal of Controlled Release*, **1992**, 19, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (e.g. as a solution or as an emulsion) were ineffective (Weiner et al., *Journal of Drug Targeting*, **1992**, 2, 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal

formulation was superior to aqueous administration (du Plessis et al., *Antiviral Research*, 1992, 18, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu et al. *S.T.P. Pharma. Sci.*, 1994, 4, 6, 466).

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G<sub>M1</sub>, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen et al., *FEBS Letters*, 1987, 223, 42; Wu et al., *Cancer Research*, 1993, 53, 3765). Various liposomes comprising one or more glycolipids are known in the art.

Papahadjopoulos et al. (*Ann. N.Y. Acad. Sci.*, **1987**, 507, 64) reported the ability of monosialoganglioside  $G_{M1}$ , galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were  
5 expounded upon by Gabizon et al. (*Proc. Natl. Acad. Sci. U.S.A.*, **1988**, 85, 6949). U.S. Patent No. 4,837,028 and WO 88/04924, both to Allen et al., disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside  $G_{M1}$  or a galactocerebroside sulfate ester. U.S. Patent No.  
10 5,543,152 (Webb et al.) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-sn-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim et al.).

Many liposomes comprising lipids derivatized with one  
15 or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto et al. (*Bull. Chem. Soc. Jpn.*, **1980**, 53, 2778) described liposomes comprising a nonionic detergent, 2C<sub>12</sub>15G, that contains a PEG moiety. Illum et al. (*FEBS Lett.*, **1984**, 167, 79) noted  
20 that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (e.g., PEG) are described by Sears (U.S. Patent Nos.  
25 4,426,330 and 4,534,899). Klibanov et al. (*FEBS Lett.*, **1990**, 268, 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume et al.  
30 (*Biochimica et Biophysica Acta*, **1990**, 1029, 91) extended such observations to other PEG-derivatized phospholipids, e.g., DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their

external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by

5 Woodle *et al.* (U.S. Patent Nos. 5,013,556 and 5,356,633) and Martin *et al.* (U.S. Patent No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 (both to Martin *et al.*) and in WO 94/20073 (Zalipsky *et al.*) Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi *et al.*). U.S. Patent Nos. 5,540,935 (Miyazaki *et al.*) and 5,556,948 (Tagawa *et al.*) describe

15 PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

A limited number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry *et al.* discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Patent No. 5,264,221 to

20 Tagawa *et al.* discloses protein-bonded liposomes and asserts that the contents of such liposomes may include an antisense RNA. U.S. Patent No. 5,665,710 to Rahman *et al.* describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love *et al.* discloses liposomes comprising antisense

25 oligonucleotides targeted to the raf gene.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes may

30 be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, e.g. they are self-optimizing (adaptive to the shape of

35 pores in the skin), self-repairing, frequently reach their

targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum  
5 albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such  
10 as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also  
15 known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, 1988, p. 285).

If the surfactant molecule is not ionized, it is  
20 classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic  
25 esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block  
30 polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant  
35 is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates

and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl  
5 sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The  
10 quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include  
15 acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in  
*Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York,  
20 NY, 1988, p. 285).

#### Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient  
25 delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic  
30 drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

35 Penetration enhancers may be classified as belonging to one of five broad categories, i.e., surfactants, fatty

acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92). Each of the above mentioned classes of penetration enhancers are described  
5 below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution,  
10 reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration  
15 enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi et al., *J. Pharm.*  
20 *Pharmacol.*, 1988, 40, 252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid),  
25 myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprinate, tricaprinate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprinate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines,  
30 C<sub>1-10</sub> alkyl esters thereof (e.g., methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92; Muranishi,  
35 *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990,

7, 1-33; El Hariri et al., J. Pharm. Pharmacol., 1992, 44, 651-654).

Bile salts: The physiological role of bile includes  
5 the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al. Eds., McGraw-Hill, New York, 1996, pp. 934-935). Various natural bile salts, and their synthetic  
10 derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable  
15 sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucolic acid (sodium glucolate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate),  
20 taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydrofusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee et al., *Critical*  
25 *Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92; Swinyard, Chapter 39 In: *Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages 782-783; Muranishi, *Critical*  
*Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33; Yamamoto et al., J. Pharm. Exp. Ther., 1992, 263, 25; Yamashita et al., J. Pharm. Sci., 1990, 79, 579-583).

Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as  
35 compounds that remove metallic ions from solution by



forming complexes therewith, with the result that absorption of oligonucleotides through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, *J. Chromatogr.*, **1993**, 618, 315-339). Chelating agents of the invention include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate and homovanilate), *N*-acyl derivatives of collagen, laureth-9 and *N*-amino acyl derivatives of beta-diketones (enamines) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7, 1-33; Buur et al., *J. Control Rel.*, **1990**, 14, 43-51).

Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7, 1-33). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacycloalkanone derivatives (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., *J. Pharm. Pharmacol.*, **1987**, 39, 621-626).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and

other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi et al, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo et al.,  
5 PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol,  
10 pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

#### Carriers

Certain compositions of the present invention also  
15 incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (i.e., does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the  
20 bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance,  
25 can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially  
30 phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid (Miyao et al.,  
*Antisense Res. Dev.*, **1995**, 5, 115-121; Takakura et al.,  
35 *Antisense & Nucl. Acid Drug Dev.*, **1996**, 6, 177-183).

## Excipients

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrants (e.g., starch, sodium starch glycolate, etc.); and wetting agents (e.g., sodium lauryl sulphate, etc.).

Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or

solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

#### Other Components

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include but are not limited to daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptapurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed. 1987, pp. 1206-1228, Berkow et al., eds., Rahway, N.J. When used with the compounds of the invention, such chemotherapeutic agents may be used individually (e.g., 5-FU and oligonucleotide), sequentially (e.g., 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (e.g., 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. See, generally, *The Merck Manual of Diagnosis*

and Therapy, 15th Ed., Berkow et al., eds., 1987, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be  
5 used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds  
10 targeted to a second nucleic acid target. Numerous examples of antisense compounds are known in the art. Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the  
15 skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules  
20 can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and  
25 can generally be estimated based on  $EC_{50}$ s found to be effective in in vitro and in vivo animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years.  
30 Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy  
35 to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses,

ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with specificity in accordance with certain of its preferred  
5 embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

#### EXAMPLES

##### Example 1

##### 10 Nucleoside Phosphoramidites for Oligonucleotide Synthesis Deoxy and 2'-alkoxy amidites

2'-Deoxy and 2'-methoxy beta-cyanoethyl-diisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling  
15 VA). Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the  
20 wait step after pulse delivery of tetrazole and base was increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published methods [Sanghvi, et. al., *Nucleic Acids*  
25 *Research*, 1993, 21, 3197-3203] using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

##### 2'-Fluoro amidites

##### 30 2'-Fluorodeoxyadenosine amidites

2'-fluoro oligonucleotides were synthesized as described previously [Kawasaki, et. al., *J. Med. Chem.*,  
1993, 36, 831-841] and United States patent 5,670,633, herein incorporated by reference. Briefly, the protected  
35 nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was

synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S<sub>N</sub>2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

#### 2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropylidisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

#### 2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'-phosphoramidites.



**2'-Fluorodeoxycytidine**

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

**2'-O-(2-Methoxyethyl) modified amidites**

2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, 1995, 78, 486-504.

**2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]**

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or it can be purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C).

**2'-O-Methoxyethyl-5-methyluridine**

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH<sub>3</sub>CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH<sub>2</sub>Cl<sub>2</sub>/acetone/MeOH (20:5:3) containing 0.5% Et<sub>3</sub>NH. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product. Additional material was obtained by reworking impure fractions.

**2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine**

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH<sub>3</sub>CN (200 mL). The residue was dissolved in CHCl<sub>3</sub> (1.5 L) and extracted with 2x500 mL of saturated NaHCO<sub>3</sub> and 2x500 mL of saturated NaCl. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/hexane/acetone (5:5:1) containing 0.5% Et<sub>3</sub>NH. The pure fractions were evaporated

to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

5           **3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine**

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and  
10 acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by TLC by first quenching the TLC sample with the addition of MeOH. Upon completion of the reaction, as judged by TLC, MeOH (50 mL) was added and the mixture  
15 evaporated at 35°C. The residue was dissolved in CHCl<sub>3</sub> (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of CHCl<sub>3</sub>. The combined organics were dried with sodium sulfate and  
20 evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

25

**3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine**

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-  
30 methyluridine (96 g, 0.144 M) in CH<sub>3</sub>CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH<sub>3</sub>CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. POCl<sub>3</sub> was added dropwise, over a 30 minute period, to the stirred  
35 solution maintained at 0-10°C, and the resulting mixture

stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of NaHCO<sub>3</sub> and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

**2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine**

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH<sub>4</sub>OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH<sub>3</sub> gas was added and the vessel heated to 100°C for 2 hours (TLC showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

**N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine**

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, TLC showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL).

The residue was dissolved in  $\text{CHCl}_3$  (700 mL) and extracted with saturated  $\text{NaHCO}_3$  (2x300 mL) and saturated  $\text{NaCl}$  (2x300 mL), dried over  $\text{MgSO}_4$  and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using  $\text{EtOAc/hexane}$  (1:1) containing 0.5%  $\text{Et}_3\text{NH}$  as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

**N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite**

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in  $\text{CH}_2\text{Cl}_2$  (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (TLC showed the reaction to be 95% complete). The reaction mixture was extracted with saturated  $\text{NaHCO}_3$  (1x300 mL) and saturated  $\text{NaCl}$  (3x300 mL). The aqueous washes were back-extracted with  $\text{CH}_2\text{Cl}_2$  (300 mL), and the extracts were combined, dried over  $\text{MgSO}_4$  and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using  $\text{EtOAc/hexane}$  (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

**2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-(dimethylaminooxyethyl) nucleoside amidites**

**2'-(Dimethylaminooxyethoxy) nucleoside amidites**

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected

with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

**5'-O-tert-Butyldiphenylsilyl-O<sup>2</sup>-2'-anhydro-5-methyluridine**

O<sup>2</sup>-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. tert-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC (R<sub>f</sub> 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated sodium bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the solution was cooled to -10°C. The resulting crystalline product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

**5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine**

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-O<sup>2</sup>-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were

added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and  
5 opened. TLC (Rf 0.67 for desired product and Rf 0.82 for ara-T side product, ethyl acetate) indicated about 70% conversion to the product. In order to avoid additional side product formation, the reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a  
10 warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue was  
15 purified by column chromatography (2kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield  
20 based on starting material less pure recovered starting material was 58%. TLC and NMR were consistent with 99% pure product.

**2'-O-([2-phthalimidoxy)ethyl]-5'-t-butylidiphenylsilyl-  
25 5-methyluridine**  
5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g, 36.98mmol) was mixed with triphenylphosphine (11.63g, 44.36mmol) and N-hydroxyphthalimide (7.24g, 44.36mmol). It was then dried  
30 over P<sub>2</sub>O<sub>5</sub> under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture. The  
35 rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop.

After the addition was complete, the reaction was stirred for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a  
5 flash column and eluted with ethyl acetate:hexane (60:40), to get 2'-O-([2-phthalimidooxy)ethyl]-5'-t-butyl-  
diphenylsilyl-5-methyluridine as white foam (21.819 g, 86%).

10 **5'-O-tert-butyl-  
diphenylsilyl-2'-O-[(2-  
formadoximinooxy)ethyl]-5-methyluridine**

2'-O-([2-phthalimidooxy)ethyl]-5'-t-butyl-  
diphenylsilyl-5-methyluridine (3.1g, 4.5mmol) was dissolved in dry CH<sub>2</sub>Cl<sub>2</sub> (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added  
15 dropwise at -10°C to 0°C. After 1 h the mixture was filtered, the filtrate was washed with ice cold CH<sub>2</sub>Cl<sub>2</sub> and the combined organic phase was washed with water, brine and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The solution was concentrated to get 2'-O-(aminooxyethyl) thymidine, which was then  
20 dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was added and the resulting mixture was stirred for 1 h. Solvent was removed under vacuum; residue chromatographed to get 5'-O-tert-  
butyl-  
diphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-  
25 methyluridine as white foam (1.95 g, 78%).

**5'-O-tert-Butyl-  
diphenylsilyl-2'-O-[N,N-  
dimethylaminooxyethyl]-5-methyluridine**

5'-O-tert-butyl-  
diphenylsilyl-2'-O-[(2-  
30 formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was added to this solution at 10°C under inert atmosphere. The reaction  
35 mixture was stirred for 10 minutes at 10°C. After that the



reaction vessel was removed from the ice bath and stirred at room temperature for 2 h, the reaction monitored by TLC (5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>). Aqueous NaHCO<sub>3</sub> solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% NaHCO<sub>3</sub> (25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The residue obtained was purified by flash column chromatography and eluted with 5% MeOH in CH<sub>2</sub>Cl<sub>2</sub> to get 5'-O-tert-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine as a white foam (14.6g, 80%).

#### 2'-O-(dimethylaminooxyethyl)-5-methyluridine

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then added to 5'-O-tert-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine (1.40g, 2.4mmol) and stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>). Solvent was removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH<sub>2</sub>Cl<sub>2</sub> to get 2'-O-(dimethylaminooxyethyl)-5-methyluridine (766mg, 92.5%).

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine  
2'-O-(dimethylaminooxyethyl)-5-methyluridine (750mg,  
2.17mmol) was dried over P<sub>2</sub>O<sub>5</sub> under high vacuum overnight at  
40°C. It was then co-evaporated with anhydrous pyridine  
5 (20mL). The residue obtained was dissolved in pyridine  
(11mL) under argon atmosphere. 4-dimethylaminopyridine  
(26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg,  
2.60mmol) was added to the mixture and the reaction mixture  
was stirred at room temperature until all of the starting  
10 material disappeared. Pyridine was removed under vacuum  
and the residue chromatographed and eluted with 10% MeOH in  
CH<sub>2</sub>Cl<sub>2</sub> (containing a few drops of pyridine) to get 5'-O-DMT-  
2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).

15 5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-  
methyluridine-3'-[(2-cyanoethyl)-N,N-  
diisopropylphosphoramidite]  
5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine  
(1.08g, 1.67mmol) was co-evaporated with toluene (20mL).  
20 To the residue N,N-diisopropylamine tetrazonide (0.29g,  
1.67mmol) was added and dried over P<sub>2</sub>O<sub>5</sub> under high vacuum  
overnight at 40°C. Then the reaction mixture was dissolved  
in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-  
N,N,N<sup>1</sup>,N<sup>1</sup>-tetraisopropylphosphoramidite (2.12mL, 6.08mmol)  
25 was added. The reaction mixture was stirred at ambient  
temperature for 4 hrs under inert atmosphere. The progress  
of the reaction was monitored by TLC (hexane:ethyl acetate  
1:1). The solvent was evaporated, then the residue was  
dissolved in ethyl acetate (70mL) and washed with 5%  
30 aqueous NaHCO<sub>3</sub> (40mL). Ethyl acetate layer was dried over  
anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated. Residue obtained was  
chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-  
2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-  
cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam  
35 (1.04g, 74.9%).

**2'-(Aminooxyethoxy) nucleoside amidites**

2'-(Aminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(aminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs.

- 5 Adenosine, cytidine and thymidine nucleoside amidites are prepared similarly.

**N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-**  
10 **[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]**

- The 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine riboside. Multigram quantities of diaminopurine riboside may be purchased from Schering AG (Berlin) to provide 2'-O-  
15 (2-ethylacetyl) diaminopurine riboside along with a minor amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine by treatment with adenosine deaminase. (McGee, D. P. C., Cook, P. D., Guinasso, C. J.,  
20 WO 94/02501 A1 940203.) Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine which may be reduced to provide  
25 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine. As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may phosphitylated as usual to yield 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-  
30 dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite].

**2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside  
amidites**

2'-dimethylaminoethoxyethoxy nucleoside amidites (also known in the art as 2'-O-dimethylaminoethoxyethyl, i.e.,  
5 2'-O-CH<sub>2</sub>-O-CH<sub>2</sub>-N(CH<sub>2</sub>)<sub>2</sub>, or 2'-DMAEOE nucleoside amidites) are prepared as follows. Other nucleoside amidites are prepared similarly.

**2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine**

10 2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50 mmol) is slowly added to a solution of borane in tetrahydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. Hydrogen gas evolves as the solid dissolves. O<sup>2</sup>-,2'-anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium  
15 bicarbonate (2.5 mg) are added and the bomb is sealed, placed in an oil bath and heated to 155°C for 26 hours. The bomb is cooled to room temperature and opened. The crude solution is concentrated and the residue partitioned between water (200 mL) and hexanes (200 mL). The excess  
20 phenol is extracted into the hexane layer. The aqueous layer is extracted with ethyl acetate (3x200 mL) and the combined organic layers are washed once with water, dried over anhydrous sodium sulfate and concentrated. The residue is columned on silica gel using methanol/methylene  
25 chloride 1:20 (which has 2% triethylamine) as the eluent. As the column fractions are concentrated a colorless solid forms which is collected to give the title compound as a white solid.

30 **5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)-ethyl]]-5-methyl uridine**

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine in anhydrous pyridine (8 mL), triethylamine (0.36 mL) and dimethoxytrityl chloride  
35 (DMT-Cl, 0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction mixture is poured into water (200 mL) and

extracted with  $\text{CH}_2\text{Cl}_2$  (2x200 mL). The combined  $\text{CH}_2\text{Cl}_2$  layers are washed with saturated  $\text{NaHCO}_3$  solution, followed by saturated  $\text{NaCl}$  solution and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by silica gel chromatography using  $\text{MeOH}:\text{CH}_2\text{Cl}_2:\text{Et}_3\text{N}$  (20:1, v/v, with 1% triethylamine) gives the title compound.

**5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite**

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) are added to a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyluridine (2.17 g, 3 mmol) dissolved in  $\text{CH}_2\text{Cl}_2$  (20 mL) under an atmosphere of argon. The reaction mixture is stirred overnight and the solvent evaporated. The resulting residue is purified by silica gel flash column chromatography with ethyl acetate as the eluent to give the title compound.

**Example 2**

**Oligonucleotide synthesis**

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 h), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl

solution. Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as  
5 described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

10 Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and  
15 PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

20 Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both  
25 herein incorporated by reference.

### Example 3

#### Oligonucleoside Synthesis

Methylenemethylimino linked oligonucleosides, also  
30 identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligo-  
35 nucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are

prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides  
5 are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

10

#### **Example 4**

##### **PNA Synthesis**

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to  
15 in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, 1996, 4, 5-23. They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

20

#### **Example 5**

##### **Synthesis of Chimeric Oligonucleotides**

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be  
25 of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of  
30 the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

**[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric****Phosphorothioate Oligonucleotides**

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 ammonia/ethanol at room temperature overnight then lyophilized to dryness. Treatment in methanolic ammonia for 24 hrs at room temperature is then done to deprotect all bases and sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hrs at room temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

**[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-****(Methoxyethyl)] Chimeric Phosphorothioate****Oligonucleotides**

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.



**[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy  
Phosphorothioate]--[2'-O-(2-Methoxyethyl)  
Phosphodiester] Chimeric Oligonucleotides**

5 [2'-O-(2-methoxyethyl phosphodiester]--[2'-deoxy phos-  
phorothioate]--[2'-O-(methoxyethyl) phosphodiester]  
chimeric oligonucleotides are prepared as per the above  
procedure for the 2'-O-methyl chimeric oligonucleotide with  
the substitution of 2'-O-(methoxyethyl) amidites for the  
2'-O-methyl amidites, oxidization with iodine to generate  
10 the phosphodiester internucleotide linkages within the wing  
portions of the chimeric structures and sulfurization  
utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage  
Reagent) to generate the phosphorothioate internucleotide  
linkages for the center gap.  
15 Other chimeric oligonucleotides, chimeric oligonucleo-  
sides and mixed chimeric oligonucleotides/oligonucleosides  
are synthesized according to United States patent  
5,623,065, herein incorporated by reference.

20 **Example 6**

**Oligonucleotide Isolation**

After cleavage from the controlled pore glass column  
(Applied Biosystems) and deblocking in concentrated  
ammonium hydroxide at 55°C for 18 hours, the  
25 oligonucleotides or oligonucleosides are purified by  
precipitation twice out of 0.5 M NaCl with 2.5 volumes  
ethanol. Synthesized oligonucleotides were analyzed by  
polyacrylamide gel electrophoresis on denaturing gels and  
judged to be at least 85% full length material. The  
30 relative amounts of phosphorothioate and phosphodiester  
linkages obtained in synthesis were periodically checked by  
<sup>31</sup>P nuclear magnetic resonance spectroscopy, and for some  
studies oligonucleotides were purified by HPLC, as  
described by Chiang et al., *J. Biol. Chem.* **1991**, 266,  
35 18162-18171. Results obtained with HPLC-purified material

were similar to those obtained with non-HPLC purified material.

#### **Example 7**

##### **5 Oligonucleotide Synthesis - 96 Well Plate Format**

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format.

- 10 Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,4-dihydro-2H-benzothiole-3-one 1,1-dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyl-diisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base-protected beta-cyanoethyl-diisopropyl phosphoramidites.

- 20 Oligonucleotides were cleaved from support and deprotected with concentrated  $\text{NH}_4\text{OH}$  at elevated temperature (55-60°C) for 12-16 hours and the released product then dried in vacuo. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

#### **Example 8**

##### **30 Oligonucleotide Analysis - 96 Well Plate Format**

- The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman P/ACE™ MDQ) or, for individually prepared samples, on a commercial CE apparatus

(e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and  
5 multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

#### Example 9

##### 10 Cell culture and oligonucleotide treatment

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using,  
15 for example, PCR or Northern blot analysis. The following 5 cell types are provided for illustrative purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen. This can be readily determined by methods routine in the art, for  
20 example Northern blot analysis, Ribonuclease protection assays, or RT-PCR.

##### T-24 cells:

The human transitional cell bladder carcinoma cell  
25 line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies,  
30 Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates  
35 (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

5

A549 cells:

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

25

HEK cells:

Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

35 b.END cells:

The mouse brain endothelial cell line b.END was obtained from Dr. Werner Risau at the Max Plank Institute

(Bad Nauheim, Germany). b.END cells were routinely cultured in DMEM, high glucose (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD). Cells were  
5 routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 3000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells may be  
10 seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

#### Treatment with antisense compounds:

15 When cells reached 80% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 200  $\mu$ L OPTI-MEM™-1 reduced-serum medium (Gibco BRL) and then treated with 130  $\mu$ L of OPTI-MEM™-1 containing 3.75  $\mu$ g/mL LIPOFECTIN™ (Gibco BRL)  
20 and the desired concentration of oligonucleotide. After 4-7 hours of treatment, the medium was replaced with fresh medium. Cells were harvested 16-24 hours after oligonucleotide treatment.

The concentration of oligonucleotide used varies from  
25 cell line to cell line. To determine the optimal oligonucleotide concentration for a particular cell line, the cells are treated with a positive control oligonucleotide at a range of concentrations. For human cells the positive control oligonucleotide is ISIS 13920,  
30 **TCCGTCATCGCTCCTCAGGG**, SEQ ID NO: 1, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to human H-ras. For mouse or rat cells the positive control oligonucleotide is ISIS 15770, **ATGCATTCTGCCCAAGGA**, SEQ ID NO: 2, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with  
35 a phosphorothioate backbone which is targeted to both mouse

and rat c-raf. The concentration of positive control oligonucleotide that results in 80% inhibition of c-Ha-ras (for ISIS 13920) or c-raf (for ISIS 15770) mRNA is then utilized as the screening concentration for new  
5 oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not achieved, the lowest concentration of positive control oligonucleotide that results in 60% inhibition of H-ras or c-raf mRNA is then utilized as the oligonucleotide screening concentration in  
10 subsequent experiments for that cell line. If 60% inhibition is not achieved, that particular cell line is deemed as unsuitable for oligonucleotide transfection experiments.

#### 15 **Example 10**

##### **Analysis of oligonucleotide inhibition of FLIP-c expression**

Antisense modulation of FLIP-c expression can be assayed in a variety of ways known in the art. For example, FLIP-c mRNA levels can be quantitated by, e.g.,  
20 Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are taught in, for example,  
25 Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Northern blot analysis is routine in the art and is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume  
30 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., 1996. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's  
35 instructions.

Protein levels of FLIP-c can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-activated cell sorting (FACS). Antibodies directed to FLIP-c can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal antisera are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., 1997. Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons, Inc., 1997.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-10.16.11, John Wiley & Sons, Inc., 1998. Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., 1997. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., 1991.

### 30 **Example 11**

#### **Poly(A)+ mRNA isolation**

Poly(A)+ mRNA was isolated according to Miura et al., *Clin. Chem.*, 1996, 42, 1758-1764. Other methods for poly(A)+ mRNA isolation are taught in, for example,

Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was  
5 washed with 200  $\mu$ L cold PBS. 60  $\mu$ L lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55  $\mu$ L of lysate was  
10 transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200  $\mu$ L of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove  
15 excess wash buffer and then air-dried for 5 minutes. 60  $\mu$ L of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C was added to each well, the plate was incubated on a 90°C hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.  
20 Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

## Example 12

### 25 Total RNA Isolation

Total RNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia CA) following the manufacturer's recommended procedures. Briefly, for  
30 cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200  $\mu$ L cold PBS. 100  $\mu$ L Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100  $\mu$ L of 70% ethanol was then added to each well and the contents mixed by



pipetting three times up and down. The samples were then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 15  
5 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96™ plate and the vacuum applied for a period of 15 seconds. The Buffer RPE wash was then repeated and the  
10 vacuum was applied for an additional 10 minutes. The plate was then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by  
15 pipetting 60 µL water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60 µL water.

The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc.,  
20 Valencia CA). Essentially, after lysing of the cells on the culture plate, the plate is transferred to the robot deck where the pipetting, DNase treatment and elution steps are carried out.

### 25 **Example 13**

#### **Real-time Quantitative PCR Analysis of FLIP-c mRNA Levels**

Quantitation of FLIP-c mRNA levels was determined by real-time quantitative PCR using the ABI PRISM™ 7700 Sequence Detection System (PE-Applied Biosystems, Foster  
30 City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR, in which amplification products

are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., JOE, FAM, or VIC, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

Prior to quantitative PCR analysis, primer-probe sets specific to the target gene being measured are evaluated for their ability to be "multiplexed" with a GAPDH amplification reaction. In multiplexing, both the target gene and the internal standard gene GAPDH are amplified concurrently in a single sample. In this analysis, mRNA isolated from untreated cells is serially diluted. Each

dilution is amplified in the presence of primer-probe sets specific for GAPDH only, target gene only ("single-plexing"), or both (multiplexing). Following PCR amplification, standard curves of GAPDH and target mRNA  
5 signal as a function of dilution are generated from both the single-plexed and multiplexed samples. If both the slope and correlation coefficient of the GAPDH and target signals generated from the multiplexed samples fall within 10% of their corresponding values generated from the  
10 single-plexed samples, the primer-probe set specific for that target is deemed multiplexable. Other methods of PCR are also known in the art.

PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by  
15 adding 25  $\mu$ L PCR cocktail (1x TAQMAN<sup>TM</sup> buffer A, 5.5 mM  $MgCl_2$ , 300  $\mu$ M each of dATP, dCTP and dGTP, 600  $\mu$ M of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNase inhibitor, 1.25 Units AMPLITAQ GOLD<sup>TM</sup>, and 12.5 Units MuLV reverse transcriptase) to 96 well plates  
20 containing 25  $\mu$ L total RNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD<sup>TM</sup>, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for  
25 1.5 minutes (annealing/extension).

Gene target quantities obtained by real time RT-PCR are normalized using either the expression level of GAPDH, a gene whose expression is constant, or by quantifying total RNA using RiboGreen<sup>TM</sup> (Molecular Probes, Inc. Eugene,  
30 OR). GAPDH expression is quantified by real time RT-PCR, by being run simultaneously with the target, multiplexing, or separately. Total RNA is quantified using RiboGreen<sup>TM</sup> RNA quantification reagent from Molecular Probes. Methods of RNA quantification by RiboGreen<sup>TM</sup> are taught in Jones, L.J.,  
35 et al, *Analytical Biochemistry*, 1998, 265, 368-374.

In this assay, 175  $\mu$ L of RiboGreen™ working reagent (RiboGreen™ reagent diluted 1:2865 in 10mM Tris-HCl, 1 mM EDTA, pH 7.5) is pipetted into a 96-well plate containing 25uL purified, cellular RNA. The plate is read in a  
5 CytoFluor 4000 (PE Applied Biosystems) with excitation at 480nm and emission at 520nm.

Probes and primers to mouse FLIP-c were designed to hybridize to a mouse FLIP-c sequence, using published sequence information (GenBank accession number Y14041,  
10 incorporated herein as SEQ ID NO:3). For mouse FLIP-c the PCR primers were:

forward primer: GAGAACCCAGACCGTTGGT (SEQ ID NO: 4)

reverse primer: AGCCGTAACCGCCAAGCT (SEQ ID NO: 5) and the

PCR probe was: FAM-CCAAGCCGCCACCCTGGATGATA-TAMRA

15 (SEQ ID NO: 6) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye. For mouse GAPDH the PCR primers were:

forward primer: GGCAAATCAACGGCACAGT (SEQ ID NO: 7)

20 reverse primer: GGGTCTCGTCTCTGGAAGCT (SEQ ID NO: 8) and the PCR probe was: 5' JOE-AAGGCCGAGAATGGGAAGCTTGTCATC- TAMRA 3' (SEQ ID NO: 9) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

25 Probes and primers to human FLIP-c were designed to hybridize to a human FLIP-c sequence, using published sequence information (GenBank accession number U97075, herein incorporated as SEQ ID NO:10 and Genbank accession number U97074, herein incorporated as SEQ ID NO:11). For  
30 human FLIP-c the PCR primers were:

forward primer: TGTGCCGGGATGTTGCTATA (SEQ ID NO: 12)

reverse primer: CAGCTTACCTCTTTCCCGTAAAAT (SEQ ID NO: 13)

and the PCR probe was: FAM-TGGTTCCACCTAATGTCAGGGACCTTCTG-TAMRA

35 (SEQ ID NO: 14) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

For human GAPDH the PCR primers were:

forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO: 15)

reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO: 16) and

the PCR probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCC- TAMRA 3'

- 5 (SEQ ID NO: 17) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

#### Example 14

##### 10 Northern blot analysis of FLIP-c mRNA levels

- Eighteen hours after antisense treatment, cell monolayers were washed twice with cold PBS and lysed in 1 mL RNAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA was prepared following manufacturer's recommended
- 15 protocols. Twenty micrograms of total RNA was fractionated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc. Solon, OH). RNA was transferred from the gel to HYBOND™-N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway,
- 20 NJ) by overnight capillary transfer using a Northern/Southern Transfer buffer system (TEL-TEST "B" Inc., Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes were fixed by UV cross-linking using a STRATALINKER™ UV Crosslinker 2400 (Stratagene, Inc,
- 25 La Jolla, CA) and then probed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions.

- To detect mouse FLIP-c, a mouse FLIP-c specific probe was prepared by PCR using the forward primer
- 30 GAGAACCCCAGACCGTTGGT (SEQ ID NO: 4) and the reverse primer AGCCGTAACCGCCAAGCT (SEQ ID NO: 5). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto,
- 35 CA).

To detect human FLIP-c, a human FLIP-c specific probe was prepared by PCR using the forward primer ATGTCTGCTGAAGTCATCCAT (SEQ ID NO: 18) and the reverse primer ATTGCTGCTTGGATAACATTC (SEQ ID NO: 19). To normalize  
5 for variations in loading and transfer efficiency membranes were stripped and probed for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

Hybridized membranes were visualized and quantitated  
10 using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to GAPDH levels in untreated controls.

#### Example 15

15 **Antisense inhibition of mouse FLIP-c expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap**

In accordance with the present invention, a series of oligonucleotides were designed to target different regions  
20 of the mouse FLIP-c RNA, using published sequences (GenBank accession number Y14041, incorporated herein as SEQ ID NO: 3, GenBank accession number U97076, incorporated herein as SEQ ID NO: 20, GenBank accession number Y14042, incorporated herein as SEQ ID NO: 21, and GenBank accession  
25 number AI746738, incorporated herein as SEQ ID NO: 22). The oligonucleotides are shown in Table 1. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 1 are chimeric  
30 oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The  
35 internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine

residues are 5-methylcytidines. The compounds were analyzed for their effect on mouse FLIP-c mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments. If present, "N.D." indicates "no data".

Table 1

Inhibition of mouse FLIP-c mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	%INHIB	SEQ ID NO
136102	5'UTR	20	10	taagtggccgcttgagaggc	16	23
136103	5'UTR	20	14	gccctaagtggccgcttgag	54	24
136104	5'UTR	20	24	actctgtccggccctaagtg	0	25
136105	5'UTR	3	4	ggctctgggaaccacgagaa	76	26
136106	5'UTR	3	27	tccagtctccatccattaag	91	27
136107	Start Codon	3	67	gctctgggccatgttcagaa	64	28
136108	Coding	3	85	gacctcggcagacacagggc	89	29
136109	Coding	3	152	catctctacacaggaagagc	91	30
136110	Coding	3	199	gctatccaggagggtccctga	69	31
136111	Coding	3	204	cttaagctatccaggagggtc	80	32
136112	Coding	3	311	cctccacgggttgctttgtct	90	33
136113	Coding	3	320	gcagggtggctcctccacggtt	0	34
136114	Coding	3	363	atcagcaggaccctataatc	78	35
136115	Coding	3	453	atcttgccctctgcctgtgta	94	36
136116	Coding	3	536	ctaacaaattcaattgggtct	87	37
136117	Coding	3	609	ccttggctggactgggtgta	0	38
136118	Coding	3	613	tgctccttggctggactggg	90	39
136119	Coding	3	722	cacgggtattccacaaatctt	89	40
136120	Coding	3	771	aaagctcctgattcctggat	89	41
136121	Coding	3	812	tctgcatcctgtaagtctct	94	42
136122	Coding	3	842	caatgatcaagcagattcct	95	43
136123	Coding	3	897	tagcccagggaagtgaaggt	80	44
136124	Coding	3	983	agtcttgatgttgggccata	74	45
136125	Coding	3	990	ctgtcatagtcttgatgttg	88	46
136126	Coding	3	1018	tcctaggctcaccagaacac	92	47
136127	Coding	3	1035	atcatgctttgggagcctcc	84	48
136128	Coding	3	1053	tgaacttgatctctgcccat	98	49
136129	Coding	3	1069	caaggagaaccctgagtga	82	50
136130	Coding	3	1092	gtgaacatgttcttgacatg	45	51
136131	Coding	3	1099	gtcccccgatgaacatgttct	89	52
136132	Coding	3	1120	ccctctgagagaagggcacg	77	53

136133	Coding	3	1156	cgactcatagttctgaataa	0	54
136134	Coding	3	1266	tcagcttctgggtgagttgt	71	55
136135	Coding	3	1354	ctgggagagcttctgcagat	71	56
136136	Stop Codon	3	1516	gggttctcacgtaggagcca	69	57
136137	3'UTR	3	1734	ccgactaaggaatgtaagta	93	58
136138	3'UTR	3	1748	ctctggcaaaacatccgact	99	59
136139	3'UTR	3	1783	acaaacaatagggttatgtc	22	60
136140	3'UTR	3	1863	aatcttgaaccactatacac	89	61
136141	3'UTR	3	1952	ggtaattacttattaaatga	60	62
136142	3'UTR	3	1975	ctgaagcaatgggtacttaa	19	63
136143	3'UTR	3	2001	agaaattgggtggccaatgtg	76	64
136144	3'UTR	3	2193	caaggagtagggctcattct	99	65
136145	3'UTR	3	2201	caacctttcaaggagtaggg	66	66
136146	3'UTR	3	2209	aagcactacaacctttcaag	93	67
136147	3'UTR	3	2230	caaggtagagactgctctcc	5	68
136148	3'UTR	3	2277	ccactcactgttgtgttctt	99	69
136149	3'UTR	3	2284	agctccccactcactgttg	88	70
136150	3'UTR	3	2297	gccaaaccagggcaagctccc	92	71
136151	3'UTR	3	2307	ctgatcctgagccaaccagg	89	72
136152	3'UTR	3	2538	tcagtgcattgggtcattgt	92	73
136153	3'UTR	3	2665	aatctgttcctgccaaagaa	58	74
136154	3'UTR	3	2704	ctcatgtctccagcttcctt	96	75
136155	3'UTR	3	2731	gaattctatatcatgcaccc	0	76
136156	3'UTR	21	761	acagcatatgaaatgagatg	53	77
136157	3'UTR	21	819	taagaaattatacacattct	67	78
136158	3'UTR	21	853	atataattttgaacactact	77	79
136159	3'UTR	21	915	tcatgccagataatgggca	0	80
136160	3'UTR	21	939	ctaaaagaaagctttccaca	99	81
136161	3'UTR	21	1109	gaggtagaaggaaacaactt	96	82
136162	3'UTR	21	1142	cttaacaggaaggaggtagg	24	83
136163	3'UTR	21	1158	gggtctagattagtctcctta	81	84
136164	3'UTR	21	1198	tctgtgggtagattctctgt	99	85
136165	3'UTR	21	1215	tgtataaaaagtagacactct	45	86
136166	3'UTR	21	1254	agtctctgttcagaagagca	51	87
136167	3'UTR	21	1294	gaaacaatctcctattaact	94	88
136168	3'UTR	21	1301	ttaagtcgaaacaatctcct	96	89
136169	3'UTR	21	1363	agttcttgtctcaaaagaag	59	90
136170	3'UTR	21	1380	aggctggattacaggtaagt	93	91
136171	3'UTR	21	1446	gctgaagcaagaggaggctc	67	92
136172	3'UTR	21	1518	tttatacatcagcaagaaag	63	93
136173	3'UTR	21	1580	tttttaagaatcaggaagtt	22	94
136174	Coding	22	53	ataagtggccgcttgagagg	41	95
136175	Coding	22	56	gccataagtggccgcttgag	51	96
136176	Coding	22	96	ggagaccaccagaaaactgg	80	97
136177	Coding	22	161	agagacactaccgggcggtc	0	98
136178	Coding	22	173	agttcttgcaatagagacac	52	99
136179	Coding	22	214	aagccaacatcattgctgag	91	100



As shown in Table 1, SEQ ID NOs 24, 26, 27, 28, 29, 30, 31, 32, 33, 35, 36, 37, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 55, 56, 57, 58, 59, 61, 62, 64, 65, 66, 67, 69, 70, 71, 72, 73, 74, 75, 77, 78, 79, 81, 82, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 95, 96, 97, 99 and 100 demonstrated at least 30% inhibition of mouse FLIP-c expression in this assay and are therefore preferred. The target sites to which these preferred sequences are complementary are herein referred to as "active sites" and are therefore preferred sites for targeting by compounds of the present invention.

#### Example 16

**Antisense inhibition of human FLIP-c expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap**

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human FLIP-c RNA, using published sequences (GenBank accession number U97075, herein incorporated as SEQ ID NO:10 and Genbank accession number U97074, herein incorporated as SEQ ID NO:11). The oligonucleotides are shown in Table 2. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 2 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human FLIP-c mRNA levels by RT-PCR as described in other examples herein. Data are

averages from two experiments. If present, "N.D." indicates "no data".

**Table 2**

5 Inhibition of human FLIP-c mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB	SEQ ID NO
23283	5' UTR	10	1	tctactcgtgccgctcgtgc	50	101
23284	5' UTR	10	89	gcctaagattgaaagtatgg	0	102
23289	Coding	10	363	atagcaacatcccggcacaa	0	103
23290	Coding	10	440	ccaagtccccgacagacagc	0	104
23291	Coding	10	477	agcaggtcaaatcgccctcac	0	105
23293	Coding	10	640	tcggcccatgtaatccttca	75	106
23294	Coding	10	657	tccttgcttatcttgccctcg	52	107
23295	Coding	10	808	tgctccttgaacagactgct	55	108
23297	Coding	10	890	gtgttatcatcctgaagtta	68	109
23298	Coding	10	940	tcacatggaacaatttccaa	40	110
23299	STOP CODON	10	945	gttaatcacatggaacaatt	0	111
23300	STOP CODON	10	959	agaggcagttccatgttaat	0	112
23301	3' UTR	10	970	aatgattaagtagaggcagt	0	113
23302	3' UTR	10	985	gatttaatcattcagaatga	0	114
23303	3' UTR	10	1005	acacatttagaaaatgaaac	0	115
23285	5' UTR	11	298	gccagcaggcagtcacttc	0	116
23286	5' UTR	11	333	gtcttgagtagacagctccgg	25	117
23287	START CODON	11	375	ttcagcagacatcctactct	0	118
23288	START CODON	11	383	tggatgacttcagcagacat	N.D.	119
23292	Coding	11	667	ccaagtccccgacagacagc	0	120
23296	Coding	11	906	acttgctccctgctccttgaa	70	121
23304	Coding	11	981	cccattatggagcctgaagt	25	122
23305	Coding	11	989	ttacttctcccattatggag	0	123
23306	Coding	11	1020	agcgccaagctgttccttaa	0	124
23307	Coding	11	1111	gcttgctcttcatcttgat	30	125
23308	Coding	11	1150	cattgccaatgcaatcgatt	60	126
23309	Coding	11	1462	gctggccctctgacaccaca	40	127
23310	STOP CODON	11	1825	cgcccagccttttggtttct	0	128

23311	3' UTR	11	1872	ccctccttggcctcccaaag	0	129
23313	3' UTR	11	1968	ccacacccacacccagctaa	0	130
23315	3' UTR	11	2041	gctatgaccctgaactcctg	25	131

As shown in Table 2, SEQ ID NOs 101, 106, 107, 108, 109, 110, 121, 126, and 127 demonstrated at least 40% inhibition of human FLIP-c expression in this assay and are therefore preferred.

#### Example 17

##### Western blot analysis of FLIP-c protein levels

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 h after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to FLIP-c is used, with a radiolabelled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).

#### Example 18

##### Fluorescence activated cell sorting (FACS) analysis of TRAIL-sensitive and TRAIL-insensitive cells lines after treatment with antisense oligonucleotides to FLIP-c

FACS analysis was used to determine the relative amounts of apoptotic cells in a cell population after oligonucleotide treatment. As cells undergo apoptosis, there is an increase in the number of cells with less than 1 x N DNA content. This represents the fragmentation of the cell's genetic material, a classic marker for the induction of apoptosis. Cells undergoing necrosis do not present this phenotype.

In these experiments, HeLa and HS578T cells were plated at  $5 \times 10^4$  cells per well of a six-well dish. These cells were transfected with no oligonucleotide or with ISIS 128326 (**TCATGCCTCTCCTGCTAGAT**; a 2'-O-methoxyethyl gapmer with the 2'-O-methoxyethyls shown in bold; herein incorporated as SEQ ID NO:132), or ISIS 23296 (FLIP-c antisense oligonucleotide) at 300nM. One day after transfection, the media and the remaining cells on the dish were collected by trypsinization and centrifugation. The cell pellet was washed in PBS and fixed in 70% ethanol at 4C for 1 hour. Cells were washed again in PBS and stained with 0.5mg/ml propidium iodide so DNA content could be determined. Samples were assayed at least 30 minutes later by FACS.

15

#### **Example 19**

##### **Effects of antisense inhibition of FLIP-c on apoptosis**

ISIS 23296 and a mismatch control, ISIS 128326, were assayed for their effect on apoptosis in HS578T cells. These cells are normally TRAIL (Tumor necrosis factor-related apoptosis- inducing ligand) resistant. This ligand is known to induce apoptosis in cells by activating the caspase cascade. Cells which were either treated with 100 ng/mL of TRAIL (R&D Systems, Minneapolis, MN) or left untreated were transfected with with 300 nM ISIS 23296 and incubated overnight. Cells and media were collected the next day and analyzed by FACS (fluorescence activated cell sorting) to determine the percentage of cells in the sub G1 phase of the cell cycle (an indicator of apoptosis). Treatment with ISIS 23296 resulted in 15% of the cells undergoing apoptosis compared to 8% for the TRAIL treated cells and 7% for the control cells receiving no treatment. However, in cells treated with both ISIS 23296 and TRAIL, the level of apoptosis increased substantially to 32%. These results confirm that treatment with ISIS 23296 is capable of inducing apoptosis in HS578T cells and that

35

treatment in combination with TRAIL induces an even higher level of cell death.

#### EXAMPLE 20

##### 5    **Effects of caspase inhibitors on apoptosis induced by antisense inhibition of FLIP-c**

          In a similar experiment, HS578T cells were treated with ISIS 23296 and a control oligonucleotide, ISIS 29848, a 20-mer random oligonucleotide (NNNNNNNNNNNNNNNNNNNNNN, 10 wherein each N is a mixture of A, C, G and T; herein incorporated as SEQ ID NO: 133) and assayed for apoptosis in the presence of caspase inhibitors. If ISIS 23296 is indeed working to activate the caspase cascade, then inhibitors of caspases should block ISIS 23296-induced 15 apoptosis. The caspase inhibitors, DEVD-fmk (Clontech), a specific inhibitor of caspase 3 and IETD-fmk (Clontech), a specific inhibitor of caspase 8, were used in combination with ISIS 23296. Cells were treated with 300 nM of ISIS 23296 or control oligo alone and in combination with the 20 caspase inhibitors. These results were compared to the same treatments in combination with the TRAIL ligand.

          Caspase inhibitors were, in fact, able to reduce the ISIS 23296-induced apoptosis. Treatment with ISIS 23296 alone resulted in 21% apoptosis, whereas treatment with 25 ISIS 23296 and the caspase 8 inhibitor resulted in 14% apoptosis while treatment with ISIS 23296 and the caspase 3 inhibitor resulted in 12% apoptosis. Control treated cells showed a much lower level of 7% apoptosis.

          Cells pretreated with the TRAIL ligand showed even 30 greater reductions in apoptotic levels when treated with ISIS 23296 and the caspase inhibitors. The combination treatment of TRAIL and ISIS 23296 resulted in 14% apoptosis while treatment with the caspase inhibitors subsequent to TRAIL treatment further reduced apoptosis (5% apoptosis in 35 caspase 8 inhibitor treated cells and 10% apoptosis in caspase 3 inhibitor treated cells). These results

demonstrate that inhibitors of caspases were able to reduce both ISIS 23296-induced apoptosis and ISIS 23296 + TRAIL-induced apoptosis suggesting that antisense inhibition of FLIP-c expression is causing the activation of the caspase cascade.

#### **Example 21**

##### **Effects of antisense inhibition of FLIP-c on caspase activation**

ISIS 23296 and a control oligonucleotide, ISIS 29848, a 20-mer random oligonucleotide (NNNNNNNNNNNNNNNNNNNNNN, wherein each N is a mixture of A, C, G and T; herein incorporated as SEQ ID NO: 133) were assayed for the activation of caspases in HS578T cells by Western blotting as described in other examples herein. Cells were transfected with 300 nM ISIS 23296 and harvested one or two days after treatment. Equal amounts of protein were run on SDS-PAGE gels followed by transfer to PVDF membranes. The antibodies used included those to caspase 7 and caspase 8.

Western blotting revealed the reduction in caspase 7 levels by 60% compared to the control and the complete reduction of caspase 8 levels. The disappearance of these bands on the blot indicated their cleavage and their inability to be recognized by their respective antibodies suggesting that ISIS 23296 does activate the caspase cascade in these cells.